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TNF-α expression patterns as potential molecular biomarker for human skin cells exposed to vesicant chemical warfare agents: sulfur mustard (HD) and Lewisite (L)

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Abstract

Studies were conducted to examine the effect of two vesicant chemical warfare agents (VCWA), one of them an arsenical, on cytokine gene expression in normal human epidermal keratinocyte (NHEK) cells. We tested 2,2′-dichlorethylsulfide (sulfur mustard, military designation HD) and 2,chlorovinyldichloroarsine (Lewisite, military designation L), which have significant differences in their chemical, physical, and toxicological properties. Human tumor necrosis factor-alpha (hTNF-α) cytokine was detected by using the enzyme-linked immunosorbent assay, a protein multiplex immunoassay, *Luminex*^{100} ™, and reverse transcription-polymerase chain reaction (RT-PCR). The messenger RNA expression of hTNF-α was determined to provide a semi-quantitative analysis. HD-stimulated NHEK induced secretion of hTNF-α in a dose-dependent manner. Dose response effect of Lewisite decreased hTNF-α levels. Time-response data indicated that the maximum response for HD occurred at 24 h with an associated cytotoxic concentration of 10^{-4} mol/L. NHEK cells stimulated with 10^{-4} mol/L HD for 24 h at 37°C increased detectable levels of hTNF-α from 5 to 28 ng/ml at an index of cell viability between 85 to 93% as detected by *Luminex*^{100} ™. Our results indicated that the increased levels of hTNF-α by HD are dependent on the primary cultures, cell densities, and chemical properties of the stimulation. Lewisite under the same conditions as HD caused a reduction of hTNF-α from control levels of 1.5 ng/ml to 0.3 ng/ml after stimulation (10^{-4} mol/L), with an index of cell viability of ~34%. We analyzed the transcriptional of hTNF-α gene and found that HD (10^{-6} to 10^{-4} mol/L) activates the hTNF-α gene in cultured NHEK and that L at 10^{-6} to 10^{-4} mol/L markedly reduces the hTNF-α gene. We conclude that the pro-inflammatory mediator, hTNF-α, could be a potential biomarker for differentiating between exposure of HD or L.

Disclaimer: The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. Army or the Department of Defense, USA.

Introduction

Our objective in this ongoing study is to compile cytotoxicologic information about specific chemical warfare agents (CWA) using *in vitro* human cellular systems that are cost effective and are useful for quality control. In addition, the identification of a biomarker may provide valuable information about the signaling pathways suspected of being indicators of CWA exposure. Sulfur mustard, 2,2′-dichlorodiethyl sulfide (see Figure 1), was first used by the Germans in the First World War. It was called Hun Stoffe by the Allies and given the designation HS later shortened to H. Distilled, or nearly pure, sulfur mustard is designated HD (Medema, 1986). Exposure to aerosol droplets of HD or HD vapor produces no immediate effect. Itching, burning and inflammation in areas where it contacts skin generally begin about 4 h after exposure, followed by swelling of the tissue. After 20 to 24 h, small blisters form around the periphery of the affected area and eventually fully developed, large blisters fill with a colorless to yellow liquid. The wound may take several months to heal (Marrs et al., 1996). In contrast, exposure of the skin to Lewisite, 2-chlorovinyldichloroarsine (see Figure 1), produces immediate pain and symptoms similar to second or third degree burns. Erythema generally appears within 30 min of exposure, followed by blistering within 4 to 24 h. Relative to HD, healing occurs more rapidly, and the risk of secondary infection is less following Lewisite exposure (Marrs et al., 1996)

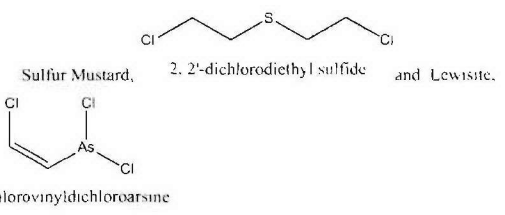

Figure 1. Chemical structure of 2,2′-dichlorodiethyl sulfide (HD) and 2-chlorovinyldichloroarsine (L).

We have selected cultures of normal human epidermal keratinocytes (NHEK) as our *in vitro* model. These cells retain many of the characteristics of the tissue from which they are isolated (Watt, 1989) and provide an appropriate experimental model for a variety of biological, biochemical, and pharmacological investigations (Wilke and Bandemir, 1989).

Cytokines play a central role in the regulation of the inflammatory skin response associated with HD-induced skin pathogenesis (Arroyo et al., 1999, 2000, 2001, 2003; Sabourin et al., 2000). Skin injuries caused by HD are complex and involve resident epidermal cells, dermal fibroblasts, and endothelial cells as well as invading leukocytes interacting with each other under the control of a network of cytokines and lipid mediators (Sidell et al., 1997). In response to vesicant stimuli, such as HD, keratinocytes produce and secrete a number of inflammatory and chemotactic cytokines, such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-alpha (TNF-α) and IL-8 (Arroyo et al., 1997, 1999, 2000, 2001, 2003). Increased expression of these mediators has been implicated in various pathological processes such as contact hypersensitivity and neoplasia (Gottlieb et al., 1988; Vasunia et al., 1994; Suzukawa et al., 2002).

Healing of wounds after trauma is a complex process relying on the coordinated action of distinct tissues, cells, and mediators (Clark, 1997; Martin, 1997). The mechanisms whereby these processes are regulated are outlined but not fully understood. Although the complete role of inflammatory cells such as macrophages and their secreted cytokines is not delineated, evidence suggests that these cells are important for wound healing and that they function primarily through the production of soluble factors including TNF-α (Leibovich, 1987). While this cytokine synthesis appears necessary for wounds to heal, excessive amounts of inflammatory cytokines are exhibited in non-healing wounds such as ulcers and/

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or inflammatory diseases (Cooney et al., 1997; Garner et al., 1993; Trengove et al., 2000). Taken together, these data suggest that while normal wound healing requires some involvement of inflammatory cytokines, abnormal wound healing may result from increased amounts of these mediators. To investigate the role of human TNF-α on NHEK stimulated with HD or L, we analyzed the secretion of hTNF-α by three different biotechniques, the enzyme-linked immunosorbent assay, a protein multiplex immunoassay, *Luminex*^{100}™, and reverse transcription-polymerase chain reaction (RT-PCR). We report that HD in cultured NHEK activates hTNF-α gene and that L at 10^{-6} to 10^{-4} mol/L markedly reduces hTNF-α expression. This suggests that the up and down regulation of hTNF-α in human skin is a unique indicator to identify and differentiate between exposures to these VCWA.

Materials and methods

Reagents

Sulfur mustard and Lewisite (5 µl in 10 ml keratinocyte growth medium (KGM™), 0.4×10^{-4} mol/L) were acquired from the US Army Soldier Biological Chemical Command (Aberdeen Proving Ground, MD, USA). The purity of HD and L was verified by nuclear magnetic resonance (NMR) to be greater than 98% (data not shown). Appropriate stock solutions of each agent (5 µl in 10 ml KGM™, 0.4×10^{-4} mol/L) were prepared and stored frozen (–80°C) in a two-phase, stable configuration until used. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was obtained from Sigma® Chemical Corp., St. Louis, MO, USA.

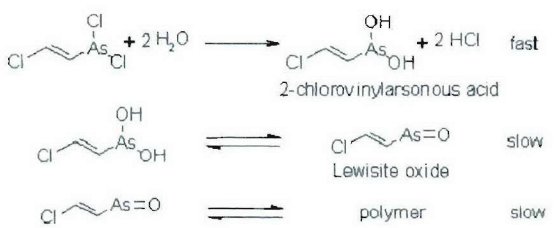
Cell culture and chemical treatments

Cryopreserved NHEK cells (Clonetics®, Bio-Whittaker, Inc., Walkersville, MD, USA) obtained from the skin of adults were cultured as described previously (Arroyo et al., 1999). These NHEK cells were grown in keratinocyte basal medium (Boyce et al., 1985) at 15×10^{-5} mol/L calcium, supplemented with 5 mg/ml insulin, 0.1 ng/L recombinant epidermal growth factor, 0.4% bovine pituitary extract, 0.5 mg/ml hydrocortisone, 50 mg/ml gentamicin and 50 ng/ml amphotericin-B. The second passage keratinocytes were subcultured in 150 cm^2 flasks at a seeding density of $\sim 2.5 \times 10^3$ cells per cm^2 in KGM™ for seven days.

When NHEK reached a desired density of $\geqslant 80\%$ in 150 cm^2 flasks, the cells were submitted to VCWA as previously reported (Arroyo et al., 2003). The cells were standardized by trypsinising and replated at a particular cell density the day before treatment with an agent. Secretion levels of hTNF-α on cell supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Arroyo et al., 1995, 1997, 1999, 2000, 2001, 2003).

Sulfur mustard (HD) and Lewisite (L) exposure

Lewisite is actually a complex mixture of several compounds, all of which occur as *cis*- and *trans* isomers of the chemical agent grade Lewisite; the 2-chlorovinylarsonous-dichloride isomer (Figure 1) generally predominates (Compton, 1988; Arroyo data not published). The hydrolysis of Lewisite proceeds according to the following scheme:



The initial hydrolysis reaction is rapid relative to formation of the 2-chlorovinylarsenous acid/Lewisite oxide equilibrium mixtures. L is relatively rapidly hydrolyzed compared with HD (Waters et al., 1950). In these studies, different doses of the chemical warfare agents were employed to determine the range of concentrations that inhibits the synthesis of TNF-α at different stages.

For ELISA experiments: NHEK in 150 cm^2 culture flasks containing fresh KGM™ media were exposed to graded doses of HD or L per flask. The culture flasks were maintained in a safety chemical fume hood for 1 h at room temperature to allow venting of volatile VCWA and then transferred to a CO_2 incubator at 37°C for an additional 24 h. After the indicated incubation time, cells were centrifuged, the supernatant media were removed, and final dilutions were made as described (Arroyo et al., 2003). Control experiments carried out with KGM™ containing graded doses of HD or L indicate that the inclusion of these VCWA for 24 h at 37°C does not influence the ELISA kit or immunoassay experiments.

For experiments performed using 96-well plates, VCWA were diluted in cell-culture media to produce indicated final concentrations, and then added to each well. The well plates were maintained at room temperature in a chemical fume hood for approximately 1 h and transferred to a CO_2 humidifier at 37°C until a particular desired incubation time was reached. After the specific incubation time, clonogenic assays were performed.

Enzyme-linked immunosorbent assay (ELISA)

ELISA experiments were performed as described in the manufacturer's literature. Human TNF-α immunoassays supplied by BioSource International, Inc. (Cytoscreen™ Immunoassay Kit, Camarillo, CA, USA) were used for the determination of soluble hTNF-α in cell supernatants. Levels of hTNF-α were determined from cell supernatants of NHEK control, HD-stimulated and L-stimulated. The optical density was measured using a microplate reader as previously documented (Arroyo et al., 2003). The absorbance of each well was read at 450 ± 10 nm, and a standard curve was constructed to quantify hTNF-α concentrations in the cell supernatant samples.

Cell viability

Human skin cells were cultured in 96-well plates and viability was measured spectrophotometrically in an MTT-assay. The MTT-based colorimetric assay used to quantify cellular growth and survival had been described by Mosmann (1983) and had been modified as a cell-mediated cytotoxicity assay by van de Loosdrecht et al. (1994). Monolayers of human skin cells were treated with increasing concentrations of HD or L for 24 h, after which time the cell viability was determined by the colorimetric MTT assay. Briefly, cells grown in a 96-well tissue culture plate are incubated with the yellow MTT solution, final concentration 0.5 mg/ml (MTT Kit, Roche Diagnostics GmbH, Mannheim, Germany) for approximately 4 h. Formazan crystals were solubilized by adding the solubilization solution of the kit and incubated in the plates overnight in a humidified atmosphere (37°C, 6.5% CO_2). The solubilized formazan product was quantified spectrophotometrically using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) attached to an IBM PC/XT for data manipulation at a wavelength of 570 ± 10 nm and a reference wavelength of 650 nm. Background absorbance values were measured in wells containing similar media without cells. An increase in the number of living cells directly correlated to the amount of purple formazan crystals formed, as monitored by the absorbance. Each experiment was performed with eight independent replicates and repeated four times.

Luminex^{100} ™ analysis system and multiplex antibody reagent kits

The general multiplex assay protocol is a sandwich immunoassay system employing beads. This novel method allowed the simultaneous measurement of 10 different biomarkers (i.e., human cytokines/ chemokine/ growth factors) and was designed to work in conjunction with the *Luminex* LabMAP™ system. This technology involved the use of a *Luminex^{100}* ™ analyzer and fluorescent encoded microspheres. Briefly, beads, buffers, and samples (including standards of known biomarker content, control specimens and unknown) were pipetted into the wells of a filter-bottom microplate (MultiScreen™-BV (1.2 μm hydrophilic, low protein binding Durapore® membrane) Millipore Corporation, Bedford, MA, USA). During the first incubation, the specific biomarker binds to the immobilized (capture) antibody on one site. After washing, a biotinylated antibody specific for a different site on the same biomarker was added. During the second incubation, this antibody binds to the immobilized biomarker captured during the first incubation. After removal of the excess second antibody, streptavidin-R-phycoerythrin (SAV-RPE), a fluorescent protein was added. The SAV-RPE binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound dye, the fluorescent materials bound to beads were quantified. The intensity of the fluorescence should be directly proportional to the concentration of biomarker present in the original specimen.

RT-PCR experiments

Approximately $5–10 \times 10^6$ cultured NHEK cells were collected, placed in 2.0 ml centrifuged tube and homogenized in a small volume of TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA). The total cellular RNA was extracted according to the manufacturer's instructions described in TRIzol® Reagent protocol.

An RNA agarose gel was prepared using 1% agarose (0.5 g) in a 1 × Na_2PO_4 buffer. Two μl of 10 × or 6 × gel-loading dye was used followed by adding 2 μl or 5 μl of RNA samples. The gel was run at 100 V until the dye had migrated ~3/4 of the way down the gel, ~35–40 min. The bands on the gel were visualized by using the GeneGenius Image Analysis System and analyzed by the GeneSnap Program (both from Syngene, Inc., Frederick, MD, USA).

The conversion of total RNA to cDNA was completed using the Ambion® protocol (Armored RNA®, Ambion Inc., Austin, TX, USA) for reverse transcription of RNA. RNA concentrations and PCR cycles were titrated to establish standard curves, to document linearity, and to permit semi-quantitative analysis of signal strength as described by Wilmer et al. (1994) and Kayama et al. (1995). When the RT-PCR reaction was completed, the PCR products were visualized by UV illumination following electrophoresis through 1% or 1.5% agarose (UltraPure, Sigma® Chemical Corp., St. Louis, MO, USA) at 100 V for ~35 min and staining in Tris-borate-EDTA buffer (89 mmol/L boric acid, 2.5 mmol/L EDTA, pH 8.2) containing 0.5 μg/ml ethidium bromide. Gels were scanned using the GeneGenius Image analysis system and analyzed using the GeneSnap Program.

Competitive PCR

Quantitative analysis of hTNF-α mRNA transcripts was performed by competitive PCR using serial dilution of cDNA fragments containing complementary sequences for the hTNF-α primer (Armored RNA® Competicon, Ambion® Inc., Austin, TX, USA). The concentration where the ratio is approximately one represents the amount of target mRNA

transcripts in the initial sample and was derived by linear regression analysis (Germolec et al., 1997).

Data analysis

Data shown are representative of at least three separate experiments. Statistical significance was determined by the RS/1 Multicomparison (Bolt, Beranek and Newman, Cambridge, MA, USA) procedure using the Wilkes-Shapiro test for normality and Dunnett's test for multiple comparisons with a common control group. When variance was nonhomogeneous, multiple comparisons utilizing the Bonferroni adjustment of the Student t were performed (Ryan, 1989). Statistically significant differences were reported when $*p < 0.05$.

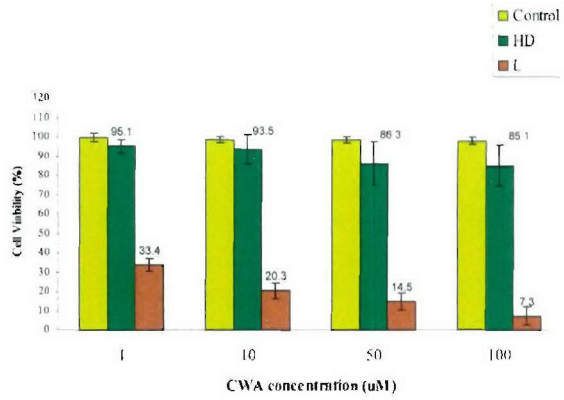


Figure 2. Cytotoxicity effects of HD and L on NHEK cells. NHEK in exponential phase growth (2×10^3 cells/well) were cultured with different concentrations of HD or L. After removal HD or L, surviving cell numbers were determined indirectly by MTT dye reduction as described in the Materials and Methods section. The reported values are percentage of cell viability for eight independent experiments. Values are means $\pm$ SD for eight independent trials (n =8).

Results

The cytotoxicity effects caused by HD or L (10^{-6} to 10^{-4} mol/L) were measured on normal human epidermal keratinocytes using MTT clonogenic assay over a period of 24 h at 37°C. The results are illustrated in Figure 2. Low levels of L (10^{-6} to 10^{-5} mol/L) produced a modest but significant decrease in keratinocyte viability while higher levels of L (10^{-4} mol/L), associated with cytotoxicity, significantly decreased survival growth 34% (Figure 2). Cell viability experiments (flow cytrometric analysis and MTT-assay) of controls (non-stimulated) and HD-stimulated cells showed that the cell viability for controls was greater than 95% of surviving cells and approximately 85% or lower with 10^{-4} mol/L HD under similar culture conditions (Figure 2).

We investigated the induction of hTNF-α by HD or L on NHEK. Dose and time responses were generated. Cells treated with HD display markedly increased hTNF-α in a dose and time dependent manner as shown in Figure 3. Stimulation with L blocked hTNF-α at higher concentrations associated with cytotoxocity (10^{-5} to 10^{-4} mol/L) (Figures 3 and 4). Figure 3 illustrates viability on NHEK grown in the presence of HD or L (10^{-4} mol/L) for 24 h at 37°C as determined by flow cytometric techniques (Figure 3A) and by MTT clonogenic assay (Figure 3B). Typical representation of hTNF-α secretion detected by ELISA is also provided in Figure 3C.

As shown in Figure 4B, stimulation with HD (10^{-4} mol/L), resulted in an initial elevation of hTNF-α, which reached a maximum by 24 h, while levels of L induction of hTNF-α are constantly decreased as a function of time.

After assessing the levels of hTNF-α in supernatants from HD-stimulated or L-stimulated as a function of concentrations and time, we selected 10^{-4} mol/L and 24 h as the concentration and time associated with cytotoxicity effects. This concentration and post exposure time are needed to produce the observed effects in the skin of VCWA casualties (Vogt et al., 1984; Papirmeister et al., 1991; Marrs et al., 1996; Sidell et al., 1997). The secretion

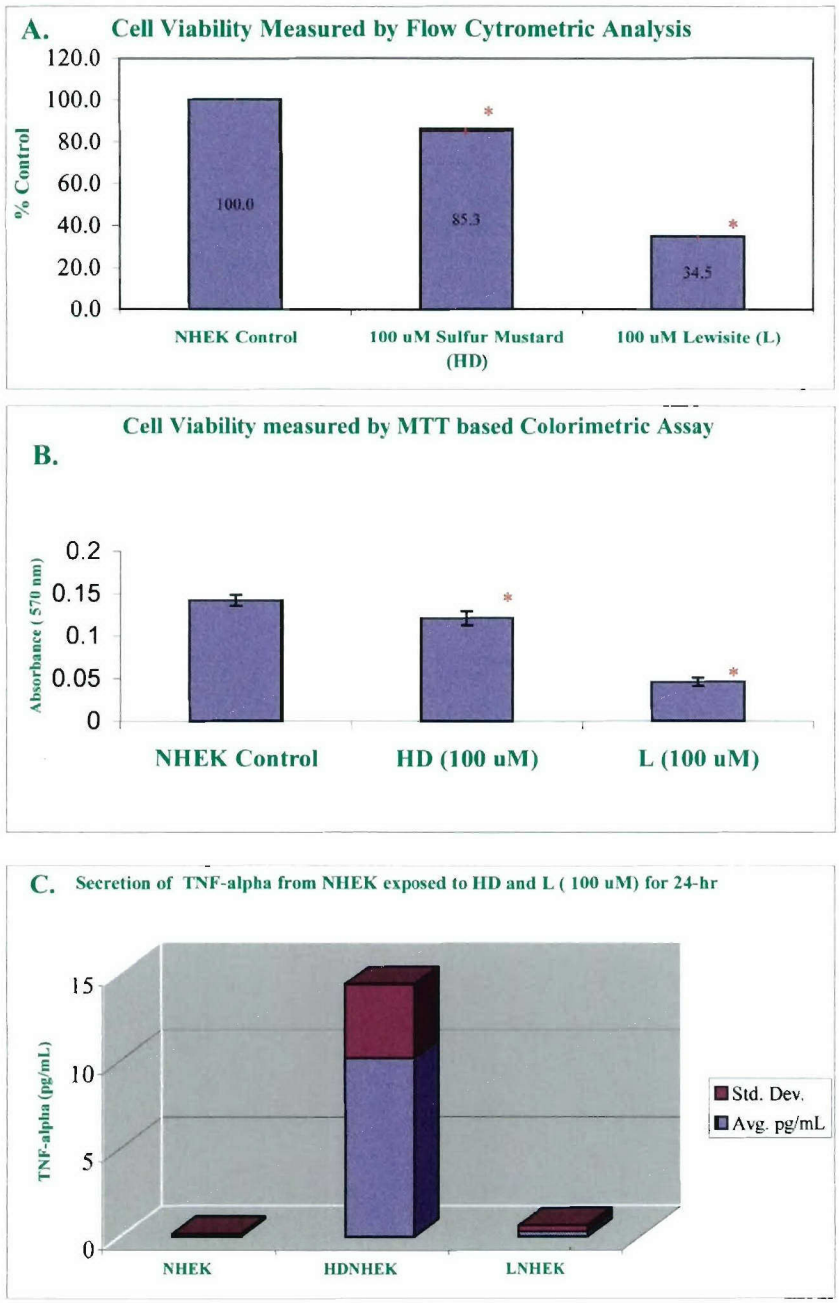
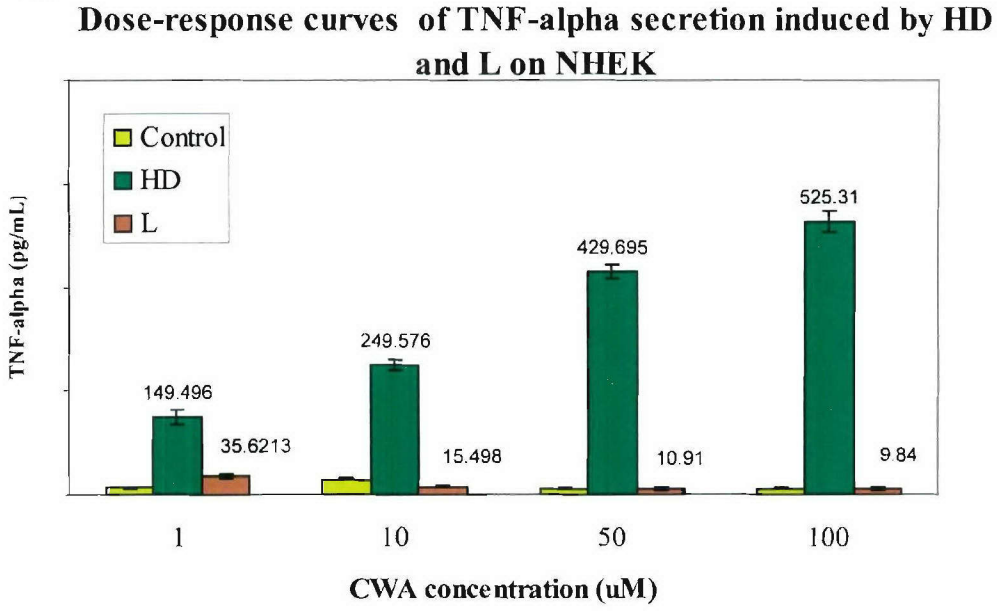


Figure 3. Viability and cytokine secretion from normal human epidermal keratinocytes (NHEK) grown in the presence of HD and L. 10^{-4} mol/L, for 24 h at 37°C. (A) Cell viability as determined by flow cytometric techniques. (B) Cell viability as determined by MTT clonogenic assay. The increase in absorbance directly correlates to the number of living cells. (C) Human TNF-α concentrations were determined by ELISA (Cyto*screen*US™ ultrasensitive. BioSource International. Camarillo. CA. USA). Results are expressed as the mean for quadruplicate determinations from one of five representative experiments. Each bar represents the mean of quartet cultures. Statistically significant differences were reported when $*p < 0.05$.

A.



B.

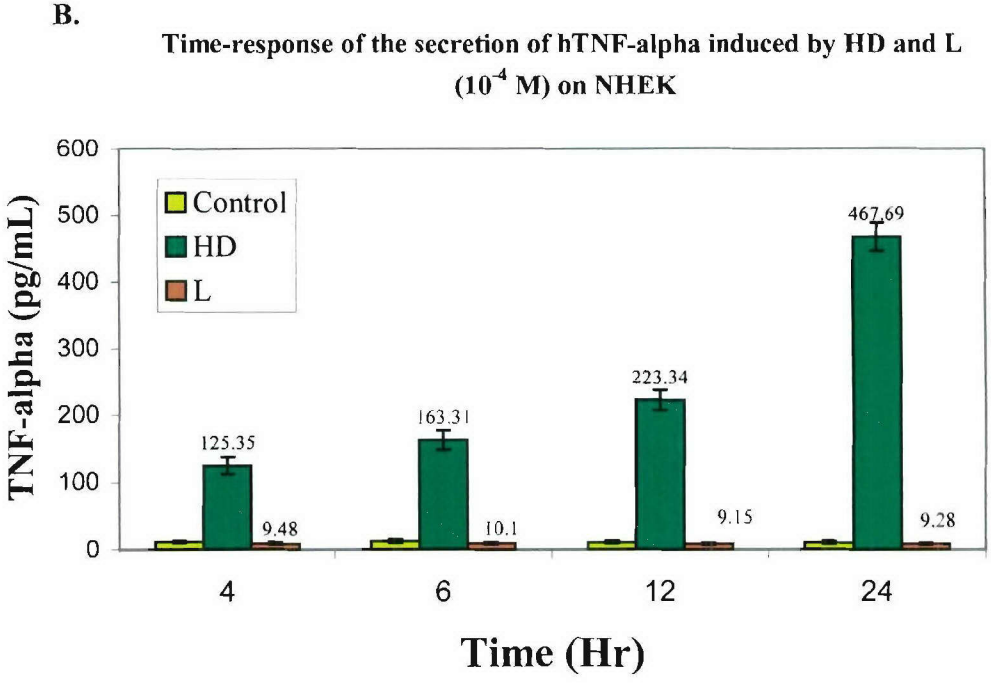


Figure 4. (A) Dose-response effects on the secretion of hTNF-α by NHEK after stimulation with HD or L (10^{-6} to 10^{-4} mol/L) for 24 h at 37°C. ELISA was assay using Cytoscreen US™ ultrasensitive commercial kit as described in Materials and Methods section. Results (mean ± SD) are expressed as pg/ml. Representative assay of seven independent experiments ($n = 7$). (B) Time-response of the secretion of hTNF-α induced by HD and L (10^{-4} mol/L) on NHEK. Triplicate cultures were used for determination of actual hTNF-α secretion. Each bar represents the mean of hTNF-α (pg/ml) ± SD. *Significantly different from control cultures at $p < 0.05$.

Table 1 Human TNF-α detected by ELISA on stimulated NHEK cell supernatants with HD and L (10^{-4} mol/L) for 24 h at 37°C

NHEK cell density (cells/ml)	NHEK control (pg/ml)	HD-stimulated 100 μmol/L (pg/ml)	S/C[a]	L-stimulated 100 μmol/L (pg/ml)	S/C[a]
6.00×10^5	4.31 ± 1.79	243.00 ± 13.50	56.38	≅0.0 ± 0.00[b]	∝
8.00×10^5	10.60 ± 1.53	249.58 ± 5.61	23.54	10.91 ± 2.45	1.03
9.90×10^5	9.73 ± 0.57	302.30 ± 23.26	31.07	≅0.0 ± 0.00[b]	∝
1.30×10^6	29.23 ± 12.44	429.69 ± 62.01	14.70	15.49 ± 6.83	0.53
7.00×10^6	120.30 ± 0.06	1060.46 ± 0.14	8.80	148.45 ± 0.14	1.2

[a]Ratio of stimulated to control NHEK.

[b]Values were not determined because were ⩽ than control equivalent to zero.

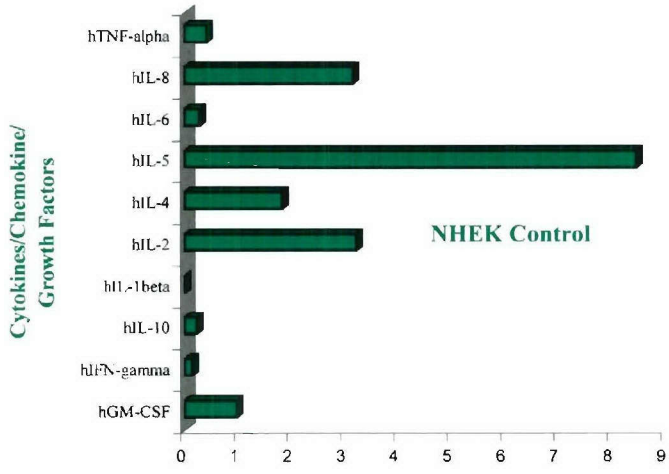
Table 2. Human TNF-α detected by *Luminex*™ on stimulated NHEK cell supernatants with HD and L (10^{-4} mol/L) for 24 h at 37°C

NHEK cell density (cells/ml)	NHEK control (ng/ml)	HD-stimulated (ng/ml)	S/C[a]	L-stimulated (ng/ml)	S/C[a]
7.8×10^6	0.75	4.60	6.13	0.13	0.17
8.1×10^6	0.41	27.36	66.73	0.85	2.07
7.7×10^6	1.41	17.26	12.24	0.96	0.68

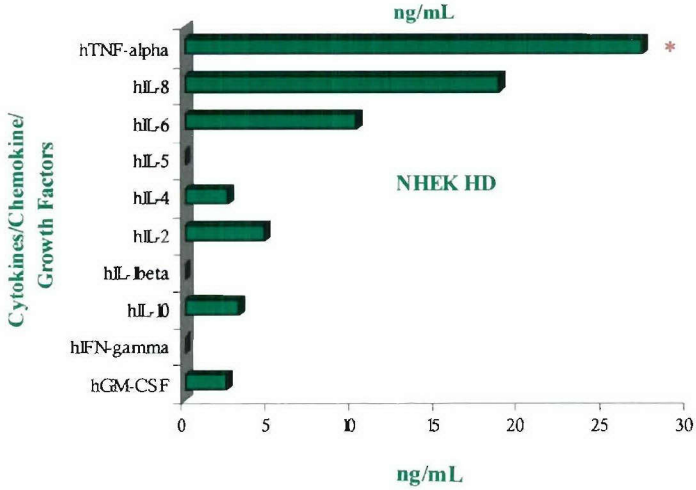
[a]Ratio of stimulated to control NHEK.

levels of hTNF-α in supernatants from NHEK stimulated with HD or L (10^{-4} mol/L) over a period of 24 h at 37°C are reported in Table 1 for ELISA assay and Table 2 for *Luminex*™. The secretion levels of hTNF-α in HD-stimulated NHEK supernatants resulted in ⩾8-fold increase from NHEK control (non-stimulated NHEK). The secretion of hTNF-α by cells stimulated with HD was considerately greater than the detected hTNF-α in supernatants from NHEK stimulated with L. In addition, the results indicate that the increased levels of hTNF-α induced by HD stimulation depend on cell densities (see Tables 1 and 2).

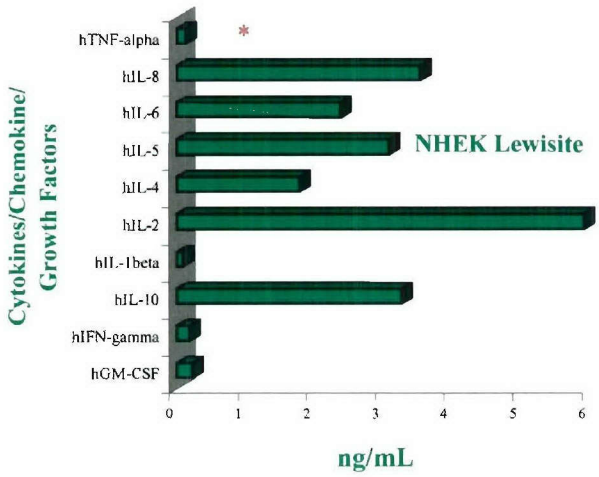
We conducted similar studies to confirm the observations reported in Table 1 and Figure 4B obtained by ELISA using a more accurate and sensitive immunoassay, *Luminex*™. This multiplex assay performs equivalent to or better than currently available ELISA assays with comparable limits of detection and with an increased dynamic range (Carson and Vignali, 1999). As shown in Figures 5B and 6B, 24 h following stimulation with HD (10^{-4} mol/L) an increase in hTNF-α levels was evident. Detectable levels of hTNF-α in NHEK cell supernatants stimulated with 10^{-4} M HD for 24 h at 37°C were augmented from 5 to 28 ng/ml at an index of cell viability between 85 to 93%. At 10^{-4} mol/L, a cytotoxic concentration, L inhibited the secretion of hTNF-α within 24 h following stimulation of keratinocyte cultures (Figures 5C and 6C). Lewisite under that same conditions as those for HD caused an inhibition of hTNF-α secretion from control levels of 1.5 ng/ml to stimulated levels of 0.3 ng/ml at an index of cell viability of ~34%. Other proinflammatory mediators, such as human interleukin-6 (hIL-6), hIL-1β or hIL-10, chemokine, hIL-8, were also secreted and



A.
Donor Information:
Age: 24
Sex: M
Race: Black
Cell Type: Abdomen
Cell Density: 8.1 x 10^6 cells/mL
Passage 3: 75-85 % Confluent



B.
Donor Information:
Age: 24
Sex: M
Race: Black
Cell Type: Abdomen
Cell Viability: 3.6 x 10^6 cells/mL
Passage 3: 75-85 % Confluent



C.
Donor Information:
Age: 24
Sex: M
Race: Black
Cell Type: Abdomen
Cell Viability: 3.3 x 10^6 cells/mL
Passage 3: 75-85 % Confluent

detected in response to HD and L. The scope of this article is focused only on the characterization of the biomarker, hTNF-α.

To provide semi-quantitative values for changes in mRNA expression of this specific cytokine, hTNF-α mRNA levels were determined in a competitive PCR assay (Figures 7 and 8). Consistent with our initial observations, there was a limited amount of endogenous expression of hTNF-α in control NHEK cell cultures, lanes 2 and 5 (Figure 7). Levels of hTNF-α mRNA were elevated after treatment with HD (10^{-6} and 10^{-5} mol/L), lanes 4 and 7 (Figure 7). There was no constitutive expression in L-treated NHEK (10^{-6} to 10^{-5} mol/L), lanes 3 and 6 (Figure 7). Regression analysis using the hTNF-α positive control showed higher intensity (>100) lane 7 (Figure 7), corresponding to a cytokine mRNA concentration of approximately 3.0×10^{-3} attomoles. Although transcription of hTNF-α was down-regulated by Lewisite stimulation (Figures 7) at 10^{-6} to 10^{-4} mol/L for 24 h, stimulation with sulfur mustard increased the expression of hTNF-α mRNA compared to control cultures (Figures 7).

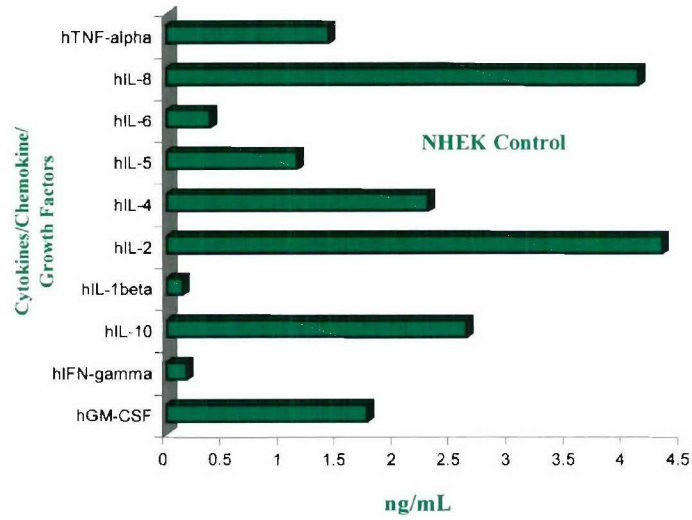
Discussion

Irritant contact dermatitis is the clinical result of sufficient inflammation arising from release of pro-inflammatory cytokines from skin cells (principally keratinocytes) in response to chemical stimuli such as vesicant agents. The three main pathophysiological changes seen are skin barrier disruption, epidermal cellular changes and cytokine release (Kan et al., 2003; Arroyo et al., 2001). Keratinocytes undergo structural changes in response to irritants and this may vary according to the type of irritant used such as HD or L. In addition, irritants disrupt the functional activity of keratinocytes.

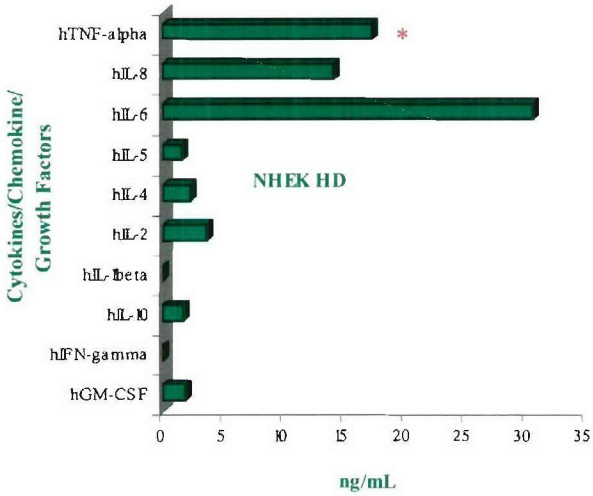
hTNF-α up-regulation has been found in response to HD (Arroyo et al., 1995, 1999, 2001). Studies of cytokine release show that irritants are heterogeneous both in cytokines released by the skin during the response, and in the quantities of cytokines released. These statements were verified by the *Luminex*^{100} ™ and ELISA data (Figures 4 and 5, Tables 1 and 2). The determined release of hTNF-α for HD was >8-fold greater than the one observed for L. HD is a particularly potent hTNF-α inducer, whereas L elicits a relatively weak hTNF-α response. hTNF-α appears to be one of the key cytokines in irritant dermatitis for HD.

Liquid arsenical vesicant, L, produces more severe lesions on the skin than liquid mustard. Contamination of the skin followed shortly by erythema, then by vesication, which tends to cover the entire area of erythema. The surrounding halo of erythema is less noticeable than with mustard blisters, although the two are often indistinguishable. Microscopically, the blister roof is slightly thicker than the mustard blister roof, consisting of almost the complete thickness of the epidermis and showing more complete coagulation necrosis and less disintegrative necrosis than that of the

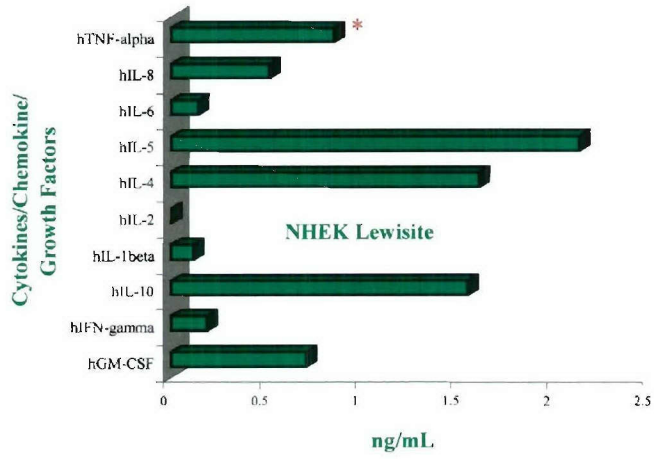
Figure 5 (opposite). Comparison of the profile of ten signaling molecules simultaneously detected by human multiplex beads in control, HD- stimulated and L-stimulated NHEK cell supernatants. NHEK were cultured to ~≥75–80% confluence as described under Materials and Methods. The supernatants were collected, and biomarker secretions were quantified by multiplex human ten-plex antibody bead kit using the Luminex^{100}™ system. Histogram bars of hTNF-α, hIL-8, hIL-6, hIL-5, hIL-4, hIL-2, hIL-1β, hIL-10, human interferon-gamma (hIFN-γ) and granulocyte macrophage-colony stimulating factor (hGM-CSF) are shown. This representation illustrated the profiling of various signaling cytokines, chemokine and growth factors simultaneously detected in control (non-stimulated) and in HD-stimulated cell supernatants of NHEK. Donor information and tissue culture conditions are indicated in the inserted description. (A) Control (non-stimulated), (B) HD-stimulated (10^{-4} mol L) for 24 h at 37 C, and (C) L-stimulated (10^{-4} mol/L) for 24 h at 37 °C. Significantly different from control cultures at $^{*}p < 0.05$ are pointed out.



A.
Donor Information:
Age: 24
Sex: M
Race: Black
Cell Type: Abdomen
Cell Density: 7.7 x 10^6 cells/mL
Passage 3: 75-80 Confluent



B.
Donor Information:
Age: 24
Sex: M
Race: Black
Cell Type: Abdomen
Cell Viability: 3.0 x 10^6 cells/mL
Passage 3: 75-80 % Confluent



C.
Donor Information:
Age: 24
Sex: M
Race: Black
Cell Type: Abdomen
Cell Viability: 2.5 x 10^6 cells/mL
Passage 3: 75-80% Confluent

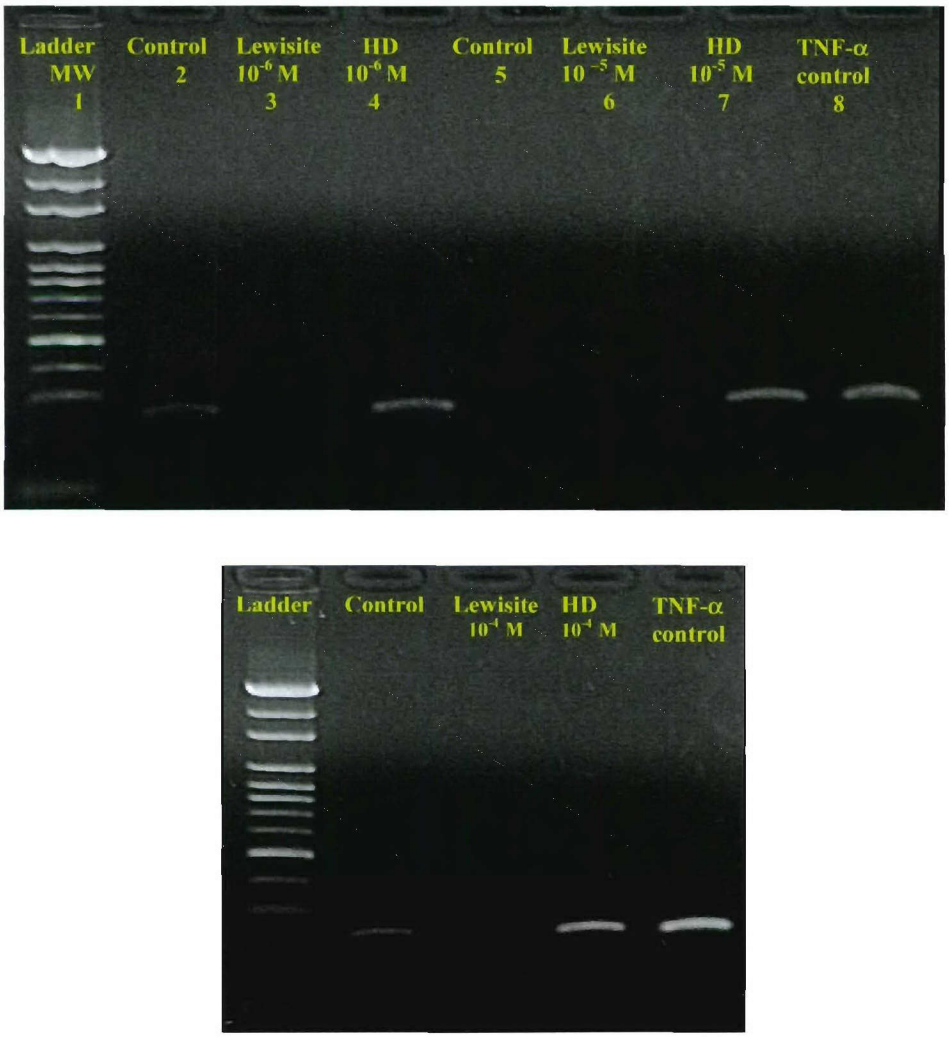


Figure 7. Lewisite inhibitions hTNF-α transcription in a dose-response manner. NHEK stimulated as follows: lane 2, control; lane 3, L (10^{-6} mol/L) for 24 h; lane 4, HD (10^{-6} mol/L); lane 5, control; lane 6, L (10^{-5} mol/L); lane 7, HD (10^{-5} mol/L) and lane 8, commercial positive control hTNF-α. Total RNA was isolated and hTNF-α mRNA levels in 0.5 μg of total RNA were determined by RT-PCR using gene-specific primers as described in Materials and Methods section. cDNA were amplified within the linear range of the gene.

Figure 6 (opposite). Immunoreactivity of NHEK culture supernatants stimulated with VCWA as determined by the multiplex immunoassay. (A) Control (non-stimulated), (B) HD-stimulated and (C) L-stimulated. VCWA concentration was 10^{-4} mol/L for 24 h at 37°C. Experimental conditions similar to the ones described in Figure 6 and in the Materials and Methods section. Results are expressed as the mean for quadruplicate determinations from one of three representative experiments. *Significantly different from control cultures at $p < 0.05$ are illustrated.

mustard blister. The yellowish blister fluid is slightly more opaque than that of the mustard blister and microscopically, contains cells that are more inflammatory. It contains a trace of arsenic but is non-toxic and non-vesicant. There is deeper injury to the connective tissue and muscle, greater vascular damage, and more severe inflammatory reaction than is exhibited in mustard burns. In large, deep, arsenical vesicant burns, there may be considerable necrosis of tissue, gangrene, and slough.

Using RT-PCR, we show that hTNF messenger RNA is induced in human skin cells but that only hTNF-α mRNA is selectively induced by HD. However, L did not induce hTNF-α mRNA. Furthermore, we confirmed the release of hTNF-α by HD from skin cells using an ELISA and *Luminex*^{100} ™ immunoassay techniques. These findings demonstrate the capacity of human skin cells to transcribe hTNF-α by exposure to toxic stimuli and to transcribe and release hTNF-α by HD exposure but not by L exposure. The concentration used (10^{-4} mol/L) has been estimated to produce the observed effects in the skin of vesicant casualties (Arroyo et al., 2003).

Furthermore, data from other investigators (Sabourin et al., 2000; Kan et al., 2003) support the involvement of TNF-α in animal models such as mouse skin and hairless guinea pigs following exposure to HD. In this work, we used a well-characterized NHEK cell system and only performed experiments using several normal donors. However, variation from baseline levels could be underestimated, even at low levels in individuals that have allergic contact dermatitis or psoriasis, it might affect baseline or inducibility.

One approach that will minimize interactions with HD or L exposure and achieves better risk estimates is the use of multiple markers in the same individual, whether using genotype, phenotype or both. The primary aim of the study was to evaluate the individual differences in cytokine release among non-stimulated controls versus cells acutely stimulated with CWA. A further purpose of the study will be to relate such patterns of cytokine release to chronic exposure to CWA, and to determine whether other cytokines, either independently or in combination with hTNF-α, can be used as "multiple markers" in VCWA. Based on our results, it is suggested that hTNF-α observed release or depletion in NHEK is associated with HD or L, respectively. The difference in TNF-α induction is not a result of reduction in viable cells but a direct correlation to the different biological responses elicited by each chemical warfare agent in viable cells. Therefore, we propose that hTNF-α, could be a good marker of vesicant chemical casualties. Finally, we recommend that a multiple marker approach to determine susceptibility to CWA should be used.

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